A Field-expedient Method for Detection of Leptospirosis Causative Agents in Rodents

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ABSTRACT

We have developed a thermal-stable, pathogenic *Leptospira* TaqMan PCR assay intended to support pathogen surveillance in reservoir populations. The assay is packaged specifically for use with a portable, ruggedized, real-time PCR thermocycler. Limit of detection was established at ≤100 fg (20 organisms). Sensitivity and specificity were 100% concordant with conventional PCR results using a broad test panel of human pathogenic and nonpathogenic *Leptospira*, genetic near neighbors, and clinically significant organisms. In blind testing using a panel (n=50) of pathogenic *Leptospira* infected and noninfected *Rattus* species samples, assay sensitivity results were 100% concordant with conventional PCR. Tests performed under field conditions using wild-collected rodent kidney extracts demonstrated the mobility of the system. During field evaluation, samples were processed and analyzed in 3 hours. Thermal stabilized reagents allowed transportation, storage, and analyses under ambient temperatures. The system provides a promising aid in leptospirosis control programs.

Routine biosurveillance and outbreak response systems are important public health tools which can facilitate prevention of infectious diseases through early detection and identification of pathogen emergence and mitigation of outbreaks through focused and timely response efforts. Rodent-borne zoonoses are a significant cause of morbidity and mortality worldwide and rapid recognition is critical to minimizing disease transmission at the local level and the spread of pathogens globally.

Leptospirosis is one of the most widespread zoonotic diseases in the world.[1-3] Genus *Leptospira* bacteria are classified into 17 species and over 200 serovars comprised of pathogenic, opportunistic, and nonpathogenic organisms.[4] *Leptospira* are transmitted by infected wild and domestic animals with rodents recognized as the most significant reservoir. Transmission to humans is by contact with infected urine in water, soil, and surfaces and through direct contact with infected animals.

The absence of a licensed vaccine against *Leptospira* and limitations in leptospirosis diagnostics and treatment drive the need for efficacious prevention and control. Surveillance of potential sources of *Leptospira* transmission serves a valuable role in leptospirosis risk assessment. Leptospirosis prevention is dependent on control of infected animals and awareness and elimination of contaminated environmental sources. To most efficiently make use of finite surveillance resources risk assessment activities must be focused on likely transmission foci and the associated environment. Analyses and risk assessment conducted in a timely manner is critical to effectively implementing prevention and control resources in an outbreak or potential outbreak situation.

Disease outbreaks often occur in developing regions and coincide with natural disasters or in war-torn areas. It is under these conditions that rapid disease surveillance, efficacious risk assessment, and appropriate and efficient use of control resources are most critical. However, *Leptospira* reference methodology by microscopic agglutination test requires up to 3 weeks for culture incubation.[5-8] As such, real-time PCR can serve as a valuable aid in surveillance and provides promise in diagnostics. Rapid and highly sensitive and specific molecular-based detection tests have been developed, however, these technologies are designed for use in a fixed laboratory infrastructure and as such are not suitable for use under austere and extreme field conditions.[7-16] In situations of an underdeveloped, damaged, or totally absent infrastructure, disease surveillance must be conducted without access to laboratory facilities, electricity, or cold-chain resources. Disruption of transportation systems and power grid are some of the obstacles that drive the need for mobile and independently operating disease surveillance systems.

We have developed a highly sensitive and specific, thermal-stable, pathogenic *Leptospira* species (LPS) PCR detection assay formatted for use with mobile, autonomously operating, field-proven, real-time PCR instrumentation.[17-20] We describe a field-expedient method for

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14. ABSTRACT

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sensitive and specific detection of leptospirosis causative agents in rodents.

MATERIALS AND METHODS

Study Site. Field-evaluation was conducted at Buri Ram Province (14° 33′ 30″ N, 102° 55′ 30″ E), Thailand, 16 to 20 August 2010. The LPS PCR assay, nucleic acid preparation reagents, and PCR instrument were transported, stored, and sample preparation and analyses conducted under ambient temperature (25°C to 33°C) and humidity (80%-100%) conditions. Staff and equipment and supplies were transported by a van to the field site. The field laboratory was set up and system operations confirmed within 2 hours. The laboratory was established in a single room of a building without environmental control using 2 tabletops (each approximately 1 m^2). Sample preparation and analyses were conducted without provisions for spatial separation or containment.

Wild-caught Rodents. Field-evaluation of the LPS assay in vivo sensitivity was conducted using a test panel of wild-caught rodent kidney tissue extracts (n=36). Sampling was conducted for 2 nights in the rice field and forest around the rural villages in 3 study sites (Chamni (14° 47′ 18″ N, 102° 50′ 30″ E), Khu Mueng (15° 16′ 18″ N, 103° 0′ 6″ E), Lahan Sai (14° 24′ 42″ N, 102° 51′ 36″ E) districts of Buri Ram province). In each site, rodent habitats were identified, and small wire live-traps (14 cm wide, 14 cm high, 30 cm long) specially fitted for rodents were set. A mixture of banana and snail was used for bait. The traps were placed in the evening (between 4 PM and 5 PM) and collected early the following morning (7 AM to 8 AM). Captured rodents were euthanized by carbon dioxide overdose.[21] Rodent kidneys and spleens were aseptically removed and extract prepared as described below. *Rattus rattus* was the most prevalent species (subspecies identification was not made). Sample extracts were transported on dry ice from the field site to the Armed Forces Research Institute of Medical Sciences laboratory for confirmation testing using a well established *Leptospira* gyrase subunit B conventional PCR assay.[15] All animal activities were approved by the Institutional Animal Care and Use Committee and conducted in an Association for Assessment and Accreditation of Laboratory Animal Care International (Frederick, MD) accredited facility and in compliance with the Animal Welfare Act (7 USC §§ 2131-2156) and other federal statutes and regulations involving animals.

Preparation of Nucleic Acid Extract. Total nucleic acid extracts were prepared from bacterial cultures, viral cultures, and rodent kidney and spleen tissues using QIAamp DNA Mini kit, QIAamp viral RNA Mini kit (QIAGEN Inc., Valencia, CA), and DNA preparation kit (Wizard Genomic DNA Purification Kit (Promega Corp, Wisconsin)) respectively. *Leptospira* DNA was quantified using the Qubit fluorometer (Life Technologies, Grand Island, NY) following the manufacturers' instructions. Extracts were stored at -70°C.

Design of PCR Probe and Primer Oligonucleotides. The LPS TaqMan PCR assay primer and probe oligonucleotide sequences may be requested from the primary author. Oligonucleotides were designed de novo by eye targeting a 132 base pair (bp) sequence of the gene encoding *Leptospira interrogans* serogroup Australis major outer membrane protein (lipl32); GenBank accession number: AY609325.1. Oligonucleotide sequences of human pathogenic *Leptospira* species were selected considering the following guidelines:

- amplicon length=75-150 bp
- oligonucleotide length=18-30 bases
- guanine and cytosine content=30%-80%
- primer melting temperature (Tm)=63°C to 67°C
- probe Tm 8°C to 10°C higher than primer Tm
- probe placement relative to primers (proximal)
- avoidance of runs of identical nucleotides to prevent mismatching and nucleotide complementarities to prevent secondary structure (hairpin-loop) formation and oligonucleotide dimerization.

Melting temperatures were quantified and the absence of significant secondary structure formation and dimerization were confirmed with PrimerExpress software (PE Applied Biosystems, Foster City, California). Primer and probe sequence heterology with genomic sequences of closely related species through diverse genera were validated by BLAST (Basic Local Alignment Search Tool) database search.[23] Primer and probe synthesis and quality control were conducted by a commercial vendor (Idaho Technology, Inc, Salt Lake City, Utah). The TaqMan probe contained 2 fluorigenic labels, a 5′ reporter dye (6-carboxyfluorescein (FAM)) and a 3′ quencher dye (6-carboxytetramethylrhodamine (TAMRA)) (Roche Molecular Diagnostics, Pleasanton, California).[24,25]

Polymerase Chain Reaction. Wet reagent LPS PCR assay optimization was conducted on the "Ruggedized" Advanced Pathogen Identification Device (R.A.P.I.D.) (Idaho Technology, Inc (ITI), Salt Lake City, Utah). Primers and probe were optimized with R.A.P.I.D. wet reagents and the optimum condition was 5 mmol/L $MgCl_2$, 400 nmol/L primers, 100 nmol/L probe. The master mix contained LPS 400 nmol/L forward and reverse primers, 100 nmol/L TaqMan probe, 200 µmol/L each dNTP, 5 mmol/L $MgCl_2$, 1×PCR buffer, 1×stabilization buffer, and Taq Polymerase:Ab: Enzyme diluent (1:1:10.5).

The optimal LPS PCR master mix formula was used for LPS assay preparation and production conducted by an ITI proprietary process. Freeze-dried LPS PCR master mix reagents only required hydration and addition of sample template prior to analysis. Assays were prepared according to the manufacturer's (ITI) instructions. A positive template control reaction was prepared using *L interrogans* serovar Bangkok at a total concentration of 1 pg template. Negative template control reactions were prepared using PCR grade water. A R.A.P.I.D. standardized PCR thermal cycling protocol consists of an initial DNA denaturation at 95°C for 3 minutes, and PCR for 45 cycles at 95°C for 0 seconds for template denaturation (sinusoidal temperature cycling) and 60°C for 20 seconds of combined annealing and primer extension.

Linearity and Limit of Detection. The linearity of the LPS freeze-dried assay was assessed in order to determine the amplification efficacy and efficiency of the PCR. These data were used to estimate limit of detection (LOD). The estimated value served as the starting point for further evaluation of LOD by replicate sample test. The correlation coefficient (R^2) of standard DNA concentrations was used to establish linearity. The slope was used to calculate amplification efficacy and efficiency using the formulas:

$$\text{Efficacy} = -1 + 10^{(-1/\text{slope})} \qquad \text{Efficiency} = 10^{(-1/\text{slope})}$$

The LOD was estimated using a standard curve produced by plotting critical threshold (Ct) values versus the logarithm of serial dilutions of *L borgpetersenii* serogroup Ballum serovar Ballum at 10 ng to 1.0 fg genomic DNA per reaction volume. The Ct values of each log DNA concentration were measured in 2 replicates. Least-squares regression analysis (performed by the R.A.P.I.D. software) plotted Ct as a function of DNA concentration. The R.A.P.I.D. software automatically calculated "best-fit" of the regression and a standard curve was established, the linear relationship between ΔPCR cycle number and ΔDNA concentration. The R^2 value was automatically adjusted near or at unity by the R.A.P.I.D. software.

The LOD was estimated as the template concentration at the lowest Ct value above background. The estimated LOD was used to conduct replicate sample testing (n=20). Replicate sample testing was conducted by 3 operators.

Rodent extracts and *Leptospira* Reference Strains. A test panel of well characterized rodent kidney extracts from sample archives was prepared consisting of 30 pathogenic *Leptospira* infected tissue extracts and 20 noninfected extracts. Kidney tissue extracts were previously prepared and confirmed positive for pathogenic *Leptospira* species by *Leptospira* gyrase subunit B conventional PCR.[8] Extracts were archived at -70°C. Prior to LPS assay sensitivity testing, template quality was confirmed using *Leptospira* gyrase subunit B conventional PCR.

Validation testing of LPS PCR assay sensitivity and specificity were conducted using a diverse panel of 24 reference serovars of *Leptospira* species consisting of 22 pathogenic and 2 nonpathogenic serovars (Table 1). Reference strains were obtained from the Department of Leptospirosis Laboratory, National Institute of Animal Health, Thailand. Cultures were grown in Ellinghausen-McCullough-Johnson-Harris medium (Difco Laboratories, Detroit, Michigan) and maintained by weekly subculture at 30°C following established methodology.[22] Reference sample quality was confirmed using *Leptospira* gyrase subunit B conventional PCR.

Non-*Leptospira* Organisms: Specificity Test Panel. Specificity testing included a panel of a well characterized nucleic acid extracts consisting of non-*Leptospira* genetic near neighbors, clinically significant organisms, and *R. rattus* and human DNA (Table 2). Organisms harboring RNA genomes underwent reverse transcription to produce genomic cDNA for testing. The intent of cDNA testing was to confirm exclusion of potential laboratory introduced crossover contaminates.

Data Analysis. Sample identification and specifications were entered electronically in the R.A.P.I.D. operating system run protocol. Analyses and results were automatically archived. The criterion for a positive result was a significant increase in fluorescence over background levels, ie, Ct, defined by an algorithm provided in the R.A.P.I.D. analytical software. The Ct is defined as the first PCR cycle with significant fluorescence when normalized against background fluorescence. Samples with a Ct of $\geq$40 were considered negative, while samples with a mean Ct of $<$40 were considered positive by R.A.P.I.D. analyses.

RESULTS

Linearity. Linear regression analyses of the LPS freeze-dried assay using *L borgpetersenii* serovar Ballum concentrations ranging from 10 ng to 1 fg of total nucleic acid (2 replicates for each of eight 10-fold dilutions) demonstrated the robustness of the assay. Amplification was linear from 10 ng to 100 fg of template concentration. Slope and best fit of correlation coefficient (R^2) and error values were performed automatically by regression analyses software included in the software package of the R.A.P.I.D. operating system. Linearity was

Table 1. Reference strains tested by *Leptospira* pathogenic spp (LPS) PCR

Serogroup	Serovar	Strain	LPS Results* (mean Ct)†
Pathogenic *L. interrogans*			
Australis	Bratislava	Jez Bratislava	35.44
Autumnalis	Autumnalis	Akiyami A	33.82
Australis	Bangkok	Bangkok-D92	34.50
Bataviae	Bataviae	Swart	35.36
Canicola	Canicola	Hond Utrecht IV	35.47
Djasiman	Djasiman	Djasiman	35.31
Hebdomadis	Hebdomadis	Hebdomadis	36.22
Icterohaemorrhagiae	Icterohaemorrhagiae	RGA	35.18
Pomona	Pomona	Pomona	35.12
Pyrogenes	Pyrogenes	Salinem	35.16
L. borgpetersenii			
Ballum	Ballum	RATTUS SP 127	34.32
Javanica	Javanica	Veldrat Bataviae 46	34.89
Mini	Mini	Sari	34.09
Sejroe	Sejroe	M84	35.33
Tarassovi	Tarassovi	Perepelitsin	35.50
L. kirschneri			
Cynopteri	Cynopteri	3522 C	35.06
Grippotyphosa	Grippotyphosa	Moskva V	34.25
L. noguchii			
Louisiana	Louisiana	LSU 1945	34.82
Panama	Panama	CZ 214	34.99
L. weilii			
Celledoni	Celledoni	Celledoni	34.77
L. santarosai			
Shermani	Shermani	1342 K	35.67
L. inadai			
Manhao	Manhao	Li 130	38.23
Nonpathogenic *L. biflexa*			
Semaranga	Patoc	Patoc I	Negative
Andamana	Andamana	CH 11	Negative
L. meyeri			
Ranarum	Ranarum	ICF	Negative

*Pathogenic *Leptospira* sample population (n=22) mean Ct=35.16, SD=0.89, CV%=0.78.
†*Leptospira* strain mean Ct value represents duplicate testing at the LOD concentration (100 fg).

Table 2. Results of negative control testing.

Species	LPS Results
Human blood	Negative
Rodent blood (*Rattus rattus*)	Negative
Escherichia coli	Negative
Shigella flexneri	Negative
Shigella sonnei	Negative
Pseudomonas aeruginosa	Negative
Klebsiella pneumoniae	Negative
Enterobacter aerogenes	Negative
Staphylococcus aureus	Negative
Staphylococcus typhimurium	Negative
Streptococcus pyogenes	Negative
Bartonella doshiae	Negative
Plasmodium falciparum	Negative
Plasmodium vivax	Negative
Japanese Encephalitis Virus (cDNA)	Negative
West Nile Virus (cDNA)	Negative
Tembusu Virus (cDNA)	Negative
Dengue Virus Serotype 1 (cDNA)	Negative
Dengue Virus Serotype 2 (cDNA)	Negative
Dengue Virus Serotype 3 (cDNA)	Negative
Dengue Virus Serotype 4 (cDNA)	Negative

quantified at slope=3.378, R^2=1.00, and error=0.0613. *Leptospira interrogans* serovar Bangkok positive template control (PTC) reaction prepared at 1 pg concentration reported fluorescence at an average Ct value of 31.85 corresponding with *L borgpetersenii* serovar Ballum 1 pg concentration average Ct value of 32.03.

Limit of Detection. The LOD was estimated at ≤100 fg or ≤20 genome equivalent (ge) based on linear regression analyses results. A total of 60 replicate R.A.P.I.D. runs at 100 fg concentration *L borgpetersenii* serovar Ballum total nucleic acid template established the LOD at ≤100 fg (20 ge). Three operators running 20 replicates reactions each over a 2-day period achieved a replicate test score of 100% (60/60). Operator 1 mean (μ) Ct values were 35.02, SD=0.51, and percent coefficient of variation values (CV%)=1.45 where n=20, SE=0.11 and 95% confidence interval (CI)=34.80-35.24. Operator 2 mean Ct values were μ=35.38, SE=0.75, and CV%=2.11 where n=20, SE=0.17, and 95% CI=35.05-35.71. Operator 3 mean Ct values were μ=35.61, SE=0.63, and CV%=1.76 where n=20, SE=0.14, and 95% CI=35.33-35.89.

Sensitivity and Specificity Testing. In LPS assay sensitivity and specificity testing with *Leptospira* reference strains, sensitivity and specificity results were 100%

concordant with *Leptospira* gyrase B conventional PCR analyses. (Table 1). Twenty-five *Leptospira* reference strains consisting of 22 pathogenic serovars were positive by LPS assay analyses and 3 nonpathogenic serovars did not report fluorescence above background. All samples were tested in duplicate at a DNA concentration of 100 fg (1×LOD). Pathogenic *Leptospira* sample population Ct values were μ=35.16, SD=0.89, and CV%=0.78 where n=22, SE=0.19, and 95% CI=34.79-35.53. Nonpathogenic serovars from the panel tested at 1 pg and 100 pg DNA concentrations (100× and 1000×LOD) reported no fluorescence above background. Inhibition of PCR was not observed at 100 pg DNA concentration (1000×LOD) using 3 pathogenic *Leptospira* serovars: *L interrogans* serogroup Australis serovar Bangkok (Ct=15.98), *L interrogans* serogroup Australis serovar Bratislava (14.34), and *L weilii* serogroup icterohaemorrhagiae serovar Sarmin (Ct=33.29). A single anomalous result occurred, *L weilii* serovar Sarmin was detected at 1 pg (Ct=39.62) but did not report fluorescence at the 100 fg LOD level. This result was not included in the statistical analyses because *L weilii* serovar Sarmin sequence is 100% homologous with primer and probe sequences and as such probable experiment error is under assessment.

Archived and wild-captured rodent kidney tissue extracts tested by the LPS assay demonstrated 100% sensitivity compared to the *Leptospira* gyrase subunit B conventional PCR assay (Table 3). Using a test panel of 50 archived rodent tissue extracts, 30 *Leptospira* infected extracts were positive by LPS assay analyses and 20 noninfected extracts did not report fluorescence above background (Table 3). Sample preparation and blind testing were conducted under controlled laboratory conditions. *Leptospira* infected rodent extract Ct values were μ=29.50, SD=3.31, and CV%=10.98 where n=30, SE=0.60, and 95% CI=28.32-30.68.

In field evaluation using a test panel of 36 wild-captured rodent tissue extracts, 4 *Leptospira* infected extracts were positive by LPS assay analyses and 32 noninfected extracts did not report fluorescence above background (Table 3). Sample preparation and testing were conducted under field conditions. Wild-captured rodent extract Ct values were μ=34.34, SD=4.83, and CV%=23.36 where n=4, SE=2.42, and 95% CI=29.61-39.07.

Specificity Testing Using Negative Control Organisms. Specificity of the LPS assay was 100% concordant with a diverse panel of well characterized non-*Leptospira* organisms (Table 2). No cross-reaction occurred with human or *Rattus* species undiluted extracts from blood or kidney tissue, respectively. Ten common infectious disease agents and Total nucleic acid extract from 10 infectious disease agents and cDNA prepared from 7 viruses were tested at a concentration of 1000×LOD. No fluorescence above background was observed for all non-*Leptospira* organisms tested.

Throughout laboratory validation testing and field evaluation, PTC reactions reported fluorescence at the expected Ct value (≈32) and negative template control reactions did not report fluorescence above background.

COMMENT

Our results clearly show that the LPS assay is a robust, portable, highly sensitive, and specific test for the detection of pathogenic *Leptospira* species In evaluation with *Leptospira* infected rodent kidney extracts, the assay proved to be sensitive with no false negative or false positive results. The stability of the assay was evidenced by the reproducibility of PTC results. Use of the assay with the R.A.P.I.D. provided a highly mobile, standalone, real-time PCR analytic system for field-deployed rodent surveillance. During field evaluation, the system was configured and normal operations confirmed in less than 2 hours. Sample processing and analyses were completed in less than 3 hours. The system is unique in its ability to fill an important public heath role as it provides rapid pathogenic *Leptospira* detection capability under austere and extreme operating conditions.

Targeting transmission risk areas and identifying preventable conditions help focus control resources. Correctly collected and interpreted data on rodent infection rate and prevalence of contaminated environment integrated with other key transmission indicators such as confirmed leptospirosis cases (where epidemiological data is available), virulence of the circulating *Leptospira* serovar, rodent infestations and population densities, reproduction rate, terrain and climatic conditions, provide for efficacious transmission risk assessment. These data collected in a spatially focused and expedient manner, augment the predictive power of field surveillance allowing decision makers to dedicate control resources for focused application of animal abatement measures, treatment of habitat, and increased public awareness. The value of animal and environmental surveillance is enhanced by field-expedient detection capability.

Limitations in leptospirosis diagnostics must be addressed. Achieving a definitive diagnosis across both the acute and immune phases of leptospirosis is challenging because clinical symptoms are easily confused with those of other common diseases.[5,26] The treatment of leptospirosis can be enhanced by rapid and highly sensitive diagnostics.[5] Antibiotics are most effective when started by day 5 of disease onset and as such early diagnosis

Table 3. Results of rodent kidney tissue testing

Samples	No. Samples	No. True Pos	No. True Neg	LPS PCR Sensitivity	gyrB PCR Sensitivity
Archived rodent extract*	50	30	20	100% (30/30+0)(100%)	100% (30/30+0)(100%)
Wild-captured rodents†	36	4	32	100% (4/4+0)(100%)	100% (4/4+0)(100%)

*Archived rodent extract (n=30) mean Ct=29.5, SD=3.31, CV%=10.98.
†Wild-captured rodent extract (n=4) mean Ct=34.34, SD=4.83, CV%=23.36.

would be beneficial in the treatment of leptospirosis.[4] However, while rats may shed up to 10^8 spirochetes per ml of urine, leptospirosis patient sample concentrations present challenges in detection limit. The presence of *Leptospira* organism/DNA can vary from very low to high levels during the acute (2-7 days) and immune (0-30 days) phases of the disease depending on the seriousness of the infection.[27] Patient urine sample concentration of *Leptospira* ranges from 10^2 to 10^4 spirochetes per ml and the asymptomatic urinary range is 10^1 to 10^3 spirochetes per ml.[28] Blood sample concentration of *Leptospira* ranges from 10^1 to 10^5 spirochetes per ml.[29] Diagnosis of leptospirosis is usually retrospective because of the length of the time required for diagnosis by microscopic agglutination test (MAT) reference methodology.[5-8] The MAT and other agglutination-based tests have been developed for more rapid and convenient diagnostics, however, these methods have limitations in specificity.[30] An approved molecular-based human diagnostic test does not currently exist that does not require confirmation testing by *Leptospira* isolation and culture. Molecular-based methodologies describing direct detection from clinical samples are not currently well represented in the literature. It is our intent to transition the LPS assay to human diagnostic use. We will address challenges in achieving efficacious PCR-based leptospirosis diagnostics by enhancing the high sensitivity and specificity of the LPS assay procedurally, adapting specialized protocols to concentrate patient samples, and the development of extraction and PCR internal positive controls.

Our results show that the LPS TaqMan assay is a field-expedient method for sensitive and specific detection of leptospirosis causative agents in rodents.

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DISCLAIMER

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